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FJ194940.1 Gene and Its Protein Product ACJ04040.1 – Potential Tumor Marker – From Protein to cDNA and Chromosomal Localization

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1. Introduction

1.1. Investigations connected with a 65 kDa tumor associated protein

1.1.1. Purification and characterization of a 65 kDa tumor associated protein

Tumorigenesis is a multistep process during which appearance of tumor-specific antigens can occur. A wide variety of transformed rodent cells secrete transformation-related proteins. In 1992, a tumor-associated phosphoprotein with molecular weight (MW) of about 65 kDa (p65) was isolated from cell culture medium containing rat transplantable hepatocellular carcinoma 1682C cell line and purified to homogeneity [1]. For purification, in the first step ammonium-sulfate precipitation and high-performance liquid chromatography on molecular-sieving and phenyl hydrophobic interaction columns were used. Next, the protein was concentrated in a Rotofor isoelectrofocusing cell and finally separated by isoelectrofocusing followed by SDS-polyacrylamide gel electrophoresis. Purification fold after the Rotofor concentration step was about 11 000. This protein had a pI of 5.8 in isoelectrofocusing gels and in SDS-PAGE migrated as a single band. The tumor origin of this 65 kDa protein was confirmed by *in vivo* labeling of hepatocellular carcinoma cells in culture with [³²P]orthophosphate or [³⁵S]methionine followed by immunoprecipitation of the p65 from cell culture medium, SDS-PAGE and autoradiography. Thin-layer chromatography (TLC) showed that the p65 molecule contains phosphotyrosine, phosphothreonine, and phosphoserine. The carbohydrate content of the purified p65 protein was confirmed by Western blot analysis with the use of biotinylated lectins. Positive reaction with concanavalin A, wheat-germ agglutinin, and *Ricinus communis*

agglutinin I was observed, which suggested the presence of D-mannose or N-acetyl-D-glucosamine, D-galactose and N-acetylglucosamine respectively [1].

In our further purification procedure of P65 protein from tissue culture of human breast carcinoma MCF-7 cells the extensive rotofor concentration step was successfully replaced with TCA precipitation. Having in hand purified p65 rat / P65 human, a comparison of both proteins isolated from different sources was performed [2]. Analysis of the amino acid compositions revealed a high degree of relatedness between the human and rat proteins. Also, N-terminal amino acid sequences of p65/P65 proteins were identical for the first 9 residues and no homology with other proteins was indicated. Amino acid sequence established for the N-terminal end of the p65 molecule was as follow: DPENVVRADT. Furthermore, peptide maps of the rat and human p65/P65 were generated by cyanogen bromide (CNBr) treatment. The cleavage of p65/P65 with CNBr resulted in four major peptides identifiable by silver staining of the Tricine SDS-PAGE prepared according to Schagger and Von Jagow 1987 [3]. The peptide patterns for p65/P65 were identical. The peptides have molecular weights of about 51, 39, 30, 19 kDa. Identical cleavage maps were obtained for rat and mouse p65. N-terminal sequence analysis of four peptides generated by CNBr treatment, resulted in short amino-acids residues 1-6, 1-10, 1-14 and 1-10, respectively, were almost identical, therefore the most probable sequences were established and their sequences were as follows: 51 kDa – TGPPWT, 39 kDa – FSLQLNSRGG, 30 kDa – REKVRLLSSARQRLR and 19 kDa - TTHNRNPKKW. High degree of homology between human and rat proteins resulted in cross-reaction of both antigens with antibodies raised in rabbits against p65 isolated from the rat cell line THC 1682C. Similar cross reactivity to polyclonal anti-rat p65 antibodies was observed for p65 from mice bearing chemically induced papillomas. On the basis of collected data it was suggested the p65 is highly conserved in different species [4].

1.2. P65 Protein in experimental models of carcinogenesis

Polyclonal antibodies raised against p65 antigen isolated from cell culture medium of transplantable hepatocellular carcinoma (THC 1682C) have been employed to monitor the carcinogenic process in multistage rodent models of liver, skin and mammary gland carcinogenesis. In the first experimental model female Sprague-Dawley rats were initiated by the single dose of 10 mg/kg body weight of N-diethylnitrosamine (DEN) in physiological saline by gastric intubation. Twenty four hours later a 2/3 of liver was removed by surgery (partial hepatectomy). Six weeks after DEN administration a part of the animals were sacrificed at one -week intervals and their livers were used for immunohistochemical analysis and their blood was analyzed for the presence of p65 by ELISA (Enzyme Linked Immunosorbent Assay). In the liver the detectable number of p65 positive minifoci were observed one week after promotion by partial hepatectomy (PH). The number of p65 positive hepatocytes increased steadily during the first three weeks after DEN/PH. Positive p65 immunoreaction was observed in the nuclei of the hepatocytes, more precisely, within the nuclear envelopes and also in the cytoplasm. Also, three weeks after DEN/PH the p65 rapidly accumulated in the blood plasma, reached a plateau by the 3rd week and then

markedly dropped by the 6th week, probably due to immune clearance. Later it increased again in parallel with the growth of p65 positive foci [4].

The second model was based on seven week old male SENCAR mice. Skin tumors were induced on the back by a single dose of 10 mmol of 7,12-dimethylbenz[a]anthracene (DMBA) and repetitive application of 1 µg of 12-O-tetradecanoylphorbol-13-acetate (TPA, carcinogenesis promoter) twice a week for 20 weeks on the shaved dorsal skin. Blood samples were randomly obtained from four mice at monthly intervals to detect p65 antigen by the use of ELISA. The p65 was detected in this system as early as 4 weeks after the 1st TPA application, with slow increases in its activity for up to 24 weeks [4].

The third model was also based on chemically {N-methyl-N-nitrosourea (NMU)}-induced mammary adenocarcinoma in rats. The presence of p65 in urine and serum of rats bearing N-methyl-N-nitrosourea-induced mammary gland adenocarcinomas were analyzed by ELISA developed on the basis of polyclonal antibodies raised against rat p65 antigen. The correlation coefficient between tumor burden and p65 concentration in urine and serum was 0.65 and 0.77, respectively. The average levels of p65 in normal urine and normal serum were 37 + 32 and 48 + 38 ng/ml, respectively. In the case of urine obtained from rats bearing mammary adenocarcinoma, the mean p65 level was 119.0 + 35.9 ng/ml and its serum level was 225.4 + 67.5 ng/ml. Sensitivity, specificity and predictive value for serum and urine marker elevation were 78.5, 70.0 and 78.5% respectively. Concentrated urinary proteins were phosphorylated *in vitro* and separated by IEF or SDS-PAGE. IEF analysis of phosphorylated proteins followed by autoradiography barely showed any radioactive bands in the urine of control rats but three major radioactive bands with pI of 5.8, 5.5 and 5.0 in the urine of adenocarcinomas-bearing animals were observed. The strongest of these bands corresponded to pI ~ 5.8 in IEF was further analyzed by SDS-PAGE and showed a MW of 65 kDa. Two lighter bands corresponded to pI 5.5 and 5.0 and MW of 50 and 41 kDa apparently representing degradation products of the p65. The 65 kDa protein with a pI of 5.8 identified in the urine of tumor-bearing rats bound to an antiphosphotyrosine monoclonal and an anti-p65 polyclonal antibody as determined by Western blot analysis [5].

Also *in vivo* phosphorylation of urinary proteins was performed by i.p. injection of [³²P]orthophosphate into control- and mammary adenocarcinoma-bearing animals, which was followed by immunoprecipitation and subsequent separation of the *in vivo*-labeled urinary proteins by SDS-PAGE. No radioactive bands were observed in the case of immunoprecipitation of control urinary proteins with the preimmune or anti-p65 IgG, as well as in experimental urinary proteins treated with preimmune serum. By using anti-p65 antibodies for immunoprecipitation of *in vivo* phosphorylated urinary proteins we were able to detect the p65 antigen only in urine of rats bearing adenocarcinomas [5].

1.3. Anti-p65 antibodies as a tool in cancer detection

Described data strongly suggest that p65 is highly conserved in various species and this phenomenon could explain why human, rat, and mouse p65 antigens cross-reacted with polyclonal antibodies raised in rabbits against p65 isolated from the rat cell line THC 1682C.

On the basis of such antibodies the ELISA procedure was developed in order to analyze the presence of p65 in blood plasma, serum and urine of rats bearing adenocarcinoma [5]. The ELISA was also tested on a limited panel of cancer patients' sera as well as sera from non-cancer patients. It was proven that serum samples obtained from patients with advanced leukemia and lung adenocarcinoma contain significantly higher levels of P65 antigen than those present in control serum [4]. Anti-p65 rabbit polyclonal antibodies were also useful in immunohistochemical studies on p65 detection in either frozen or paraffin-embedded liver sections taken from experimental animals [4].

Having in hand purified p65/P65 antigens from rat transplantable hepatocellular carcinoma THC 1682C cell line and the human breast carcinoma MCF-7 cell line the possibility to produce monoclonal antibodies (MABs) against human and rat antigen was opened [6]. A few hybridomas secreting monoclonal antibodies against p65/P65 protein were established. MABs expanded in culture medium were isolated with the use of affinity chromatography on Protein A-Sepharose, and their subclass was determined as IgG₁ by isotyping using immunodiffusion assay on 1.5% agarose gel containing goat anti-mouse subclasses of IgG and IgM. A rapid and sensitive sandwich type ELISA, with the use of purified MABs was established to measure markedly elevated amounts of p65/P65 in sera obtained from tumor-bearing animals and from cancer patients. The average level of p65 in normal rat sera was 38 ng/ml (SD 13 ng/ml), and in sera from rats bearing mammary adenocarcinomas, the average value was 1005 ± 140 ng/ml, ranging from 200 to 3400 ng/ml. In control human sera, the mean P65 level was 34 ± 35 ng/ml, while sera of patients with a variety of cancers had a 10-fold higher average P65 value of 344 ± 57 ng/ml, ranging from 50 to 2230 ng/ml. More than 80% of tested sera from adenocarcinoma-bearing rats as well as from cancer patients had p65/P65 levels elevated. Overall the assay had a sensitivity of 80.9% and specificity of 85%. Different categories of cancer such as breast, ovary and endometrium, head and neck, lung and leukemia had increased P65 levels: 83.5%, 95.0%, 81.2%, 55.5% and 88.9%, respectively [6]. The purified IgG₁ MABs with high titers and strong anti-P65 specificities were also suitable for visualization of the P65 antigen expression in tumor tissue sections [6]. Human P65 antigen has been isolated from culture medium of the breast cancer cell line (MCF-7). That is why detailed analysis of sera as well as paraffin slides from breast cancer patients was performed [7]. ELISA showed that 90.2% of cancer patients' sera were positive for P65. The average level of P65 was 446.5 ± 243.8 ng/ml and the range was from 135.2 to 958.9 ng/ml. The average P65 level in control sera was 37 ± 29.5 ng/ml. Furthermore, in the group of patients with benign breast disease P65 levels were slightly elevated above the mean, with the average P65 value of 74.0 ± 41.2 ng/ml. It was also noticed that serum P65 level correlated with the pathological state of the disease. Elevation of P65 was not obvious in patients with pathological stages 0 and 1, but in stages IIA, IIB, IIIB and IV showed a marked increase above the cut-off point $2 \pm \text{SD}$ (96ng/ml). In case of immunohistochemical analysis with the use of monoclonal antibodies against P65 80% of analyzed tissue slides were positive showing nucleocytoplasmic reaction [7].

Preliminary screening study based on P65 level determination in sera taken from patients with different categories of cancer showed increased P65 levels in the case of leukemias

(88.9%) [6]. It was the reason of P65 examination in patients with lymphocytic and granulocytic leukemia [8]. Using the anti-P65 monoclonal antibodies (MB2 and MF11) in a double-antibody sandwich ELISA the expression of the protein in sera of healthy controls, in patients with benign, non-neoplastic disease as well as in sera of patients with leukemia in different stages of development were established. The upper limit of normal P65 concentration was 115 U/ml (mean plus two standard deviations above the mean in the control group) [8]. The level of P65 was above normal in 95% of acute lymphocytic leukemia (ALL), 83% of acute myeloblastic leukemia (AML), 37% of chronic lymphocytic leukemia (CLL), and 30% of chronic myelogenous leukemia (CML). Monoclonal antibody was also used for immunocytochemical staining of isolated lymphocytes from normal peripheral blood and from blood of leukemic patients. The P65 positivity was 83% in CLL, 100% in ALL and 75% in AML patients [8]. Distribution of P65 in noncancer and cancer human sera samples base on already published results: [6-8] are presented in Table 1.

Diagnosis		Average concentration of P65 (ng/ml) in human sera \pm SD	P65-positive cases (%)
Leukemia (n=71)	ALL – acute lymphoblastic leukemia	570.6 + 86.0	95
	AML – acute myelocytic leukemia	429,5 + 71.3	83
	CLL – chronic lymphocytic leukemia	252.2 +80.6	37
	CML – chronic myelocytic leukemia	196.3 + 87.6	30
Normal (n=80)		34.8 + 34	0
Benign (n=61)		124 + 149.4	8
Breast cancer (n=132)		466.5 + 243.8	90.2
Benign (n=68)		74.0 = 41.2	20
Normal (n=112)		37.4 +29.5	10.8
Ovary/Endometrium		232 + 74	95
Head /Neck		328 + 121	81.2
Lung		328 +158	55.5

Table 1. The medium concentrations of P65 (ng/ml) in sera from normal donors and from patients with benign diseases and different malignant cancers as well as leukemias

1.4. P65-like protein

The presence of a protein antigenically related to P65 (p65-like) was also shown in fetal serum [9]. Isoelectrofocusing followed by polyacrylamide gel electrophoresis in the presence

of SDS was used for its isolation. Fractionated serum proteins after transfer onto nitrocellulose sheets were further analyzed by Western blot technique with the use of anti-P65 monoclonal antibody. Such analysis revealed that P65 protein has four isoforms. The isoforms, after isolation from polyacrylamide gels were used together for polyclonal antibody production [9]. Additional analysis of fetal serum fractions separated by electrophoresis on cellulose acetate membrane followed by immunostaining with anti-P65 monoclonal antibody revealed that the p65-like protein had a similar location to one of γ -globulin [9].

1.5. Anti-p65-like protein antibodies as a tool in cancer detection

Polyclonal antibody raised in rabbits against p65-like protein was tested by immunohistochemistry technique in breast cancer patients (frozen sections) and compared with that of anti-human-P65 monoclonal antibodies [10]. Comparison of the percentage of tumor cells showing positive immunoreaction with mono- and polyclonal antibodies showed a weak reactivity in significantly higher percentage of investigated cases 27% for MAb and 7% for PAb. On the other hand, medium and strong reactions were observed in a larger amount of cases after probing of the tumor tissue with a polyclonal antibody – 91% in comparison to 73% stained by a monoclonal antibody. In all cases positive for P65, immunohistochemical reaction was observed in the cytoplasm and in the area surrounding the nuclei [10].

Further comparative studies connected with clinical evaluation of the usefulness of polyclonal and monoclonal antibodies raised against P65/p65 protein were done by immunohistochemistry on paraffin-embedded tissue slides from breast cancer patients [11]. More than one hundred cases including infiltrating ductal breast carcinomas, fibrocystic disease and fibroadenoma were assessed immunohistochemically using monoclonal antibodies against human P65 antigen, and polyclonal antibodies against p65-like protein present in fetal serum. There were no evident differences in P65 detection between polyclonal and monoclonal antibodies, but monoclonal antibody causes more specific immunohistochemical reactions. Fibrocystic disease with large epithelioplasia that had precancerous status, gave a positive response when anti-P65 antibodies were utilized. This observation correlated with our previous data showing unique properties of anti-p65 antibodies of staining premalignant foci in the early stages of chemical carcinogenesis. In the most cases of breast cancer, a positive immunoreaction occurred with poly- and monoclonal anti-p65/P65 antibodies in the cytoplasm and/or nuclei.

The p65-like protein was also analyzed in B-chronic lymphocytic leukemia (CLL) cells as well as in normal lymphocytes, followed by their separation to nuclear, mitochondrial, and microsomal fractions by differential centrifugation. The cellular fraction of CLL and normal lymphocytes were separated by SDS-PAGE electrophoresis followed by Western blot analysis with the use of anti-p65 like polyclonal antibody [12]. P65 antigen was recognized as a predominant polypeptide in the leukemic nuclear fraction. No cross reactivity was observed with normal lymphocyte nuclear proteins in the region of 65 kDa [12].

To get a better idea about the role of the P65 protein in cancer formation and growth a more advanced study was undertaken on paraffin-embedded infiltrating ductal breast cancer tissue slides [13]. It was noticed that the percentage of positive cells with cytoplasmic expression of P65 was significantly higher in histologically more differentiated cancers (grade I and II according to Bloom & Richardson) than in grade III. The percentage of immunopositive nuclei grew with the advance of the disease and was the highest in poorly-differentiated (grade III) tumors. The tumors with P65 cytoplasmic reaction were mainly small (T1, T2), without lymph nodes (N0) and distant (M0) metastases. The straight dependence existed between P65 nucleic reaction and tumor size, metastases to lymph nodes and distant metastases. The obtained results suggested that transfer of P65 protein from cytoplasm to nuclei of the breast cancer cells is connected with more clinically advanced stages and worse prognosis for patients [13].

1.6. P65 and selected factors employed in clinical diagnosis and prognosis in breast cancer

Steroid hormone receptors (estrogen and progesterone), proliferating cell nuclear antigen (PCNA), Ki67, epidermal growth factor receptor (EGFR), oncogene c-ErbB2, tumor suppressor gene P53, anti-apoptotic gene Bcl-2 are among factors often used in clinical diagnosis and monitoring breast cancer, among other types of cancer. A computer search of Protein Sequence Database (PSD) revealed that the N-terminal peptide of human and rat P65/p65, which have been sequenced for the first 10 residues, is unique. Analysis of the amino-acid sequences of several internal peptides, generated by CNBr treatment, as described above, showed 100% identity in 6 amino-acids to c-erbA upstream of the DNA-binding domain, and 87.5% of identity in 10 amino-acids to human prostate specific antigen among other proteins [2]. On the basis of partial amino acid sequence analysis it could be suggested that P65/p65 is like the thyroid hormone receptor (THRA) or c-erbA1 and belongs to a family of nuclear receptors for various hydrophobic ligands such as steroids, vitamin D, retinoic acid and thyroid hormones. These hormones are composed of several domains important in hormone binding, DNA-binding dimerization and activation of transcription. It was shown that P65/p65 is located in nuclei of malignant cells of different cancers and this observation may support such hypothesis. It is well known that cytoplasmic receptors bind hormone and rapidly translocate it to the nucleus. This fact may explain immunostaining showing the presence of p65/P65 not only in nuclei, but also in the cytoplasm in breast cancer tissues. The presence of P65 in the cytoplasm as well as in nuclei of cancer cell may also suggest that P65 may function as a transcriptional factor.

Taking into consideration above mentioned data P65 expression was investigated in paraffin-embedded tissue slides from infiltrating ductal breast cancer specimens by immunohistochemistry using monoclonal antibodies recognizing human P65 antigen and polyclonal antibodies recognizing p65-like protein present in fetal serum in parallel with estrogen and progesterone receptors, which are very important prognostic factors in mammary gland tumors [14]. The P65/p65 expression was correlated with estrogen receptor (ER) and progesterone receptor (PR) levels. Statistically significant correlation was found

between ER or PR level and P65/p65 cytoplasmic reaction and inverse correlation with nucleic localization of P65/p65 protein [13,14]. It should be underlined that patients with high levels of ER and PR are more sensitive to antiestrogen therapy. Usually a high ER level is accompanied by a high PR level. When only one type of receptor is present, the percentage of patients sensitive to such therapy significantly decreases.

PCNA and Ki67 belong to prognostic factors for clinical evaluation of breast cancer; especially their proliferating indexes (PI-PCNA, PI-Ki67) are very useful prognostic factors. A strong correlation between PI-PCNA and PI-Ki67 was established in our studies in the group of patients suffering from infiltrating ductal breast carcinoma. High PI-PCNA was accompanied by loss of steroid receptors (ER/PR) and low level of P65/p65 antigen. Those data confirmed that appearance of P65 protein is connected to carcinogenic or tumorigenic processes and that this factor is not induced by cellular proliferation associated with non-neoplastic diseases [15]. It was also established that there is no correlation between P65 and c-ErbB2, EGFR or P53 expression. In low differentiated tumors (grade III) high P53 index and high EGFR and c-ErbB2 expression was connected with low P65 expression [16].

2. Studies of *P65/p65* gene expression at the mRNA level

2.1. Development of PCR studies of *P65/p65* gene expression in different cancers and comparison with prognostic factors

Having in hand short N-terminal amino acid sequence for whole P65/p65 molecule as well as for four peptides generated by CNBr treatment, the most probable nucleotide sequences were generated. To establish RT-PCR conditions, a forward primer was designed on the basis of N-terminal domain of whole P65 molecule and reverse primers designed on the basis of its four internal peptides. As a source of RNA for RT-PCR method optimization human promyelocytic leukemic cell line (HL-60), which secretes P65 antigen was chosen. The best primers and the best condition for the PCR were established in preliminary experiments. The best results were achieved for primers based on N-terminal sequence of whole P65 molecule and N-terminal sequence established for 51 kDa peptide. The size of the product of PCR reaction was about 150 bp long [17].

On the basis of this technique P65 gene expression was analyzed in various types of leukemia: acute myeloblastic leukemia (AML), acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL). No relationship between the expression of *P65* gene and clinical stage of leukemia was observed. The highest frequency of *P65* gene expression was found in the group of patients with CLL (average 66%). This percentage was lower in patients with acute leukemia and took out 42% in ALL and 46% in AML [17]. When *P65* gene expression was analyzed in limited groups of ALL and AML patients, where both peripheral blood and bone marrow were collected, the percentage of *P65* positivity increased to 83 and 75, respectively. Predominant presence of *P65* gene transcript in bone marrow cells may be explained by the hypothesis that investigated gene is expressed preferentially in some type of white blood cells, which do not pass through the bone marrow-blood barrier [17].

P65 gene expression was also investigated in the cases of follicular thyroid cancer and follicular adenomas and in the contrary to our earlier studies this gene was not observed in any of the analyzed follicular cancers but it was observed in 65% of follicular adenoma cases [18].

P65 gene expression was also analyzed in frozen tissue slides taken from patients diagnosed as ductal and lobular breast cancer, classified as G3, and in a limited panel of proliferative breast disease cases [19]. It was shown that *P65* gene expression is connected with small tumor size and with absence of metastases to regional lymph nodes. In contrast to *P65*, *c-ErbB2* expression was observed in patients with large tumors and with metastases to regional lymph nodes. Thus, an inverse relationship was found between these two genes (*P65* and *c-ErbB2*). Notably, the *P65* gene expression occurred in the group of proliferating breast disease cases, which were correlated with higher risk of breast cancer. In contrast, lack of *P65* was evident in cases classified as fibroadenomas [19].

Because of the biological similarity between breast and prostate adenocarcinomas and some evidence suggested that the *P65* gene may be a novel member of the superfamily of genes that encode nuclear receptors for hydrophobic ligands, *P65* gene expression was also evaluated in prostate cancer. Similarly to the investigated breast cancer cases, the expression of *P65* was observed in a significant percentage of well- and moderately-differentiated tumors [20]. *P65* gene expression was also compared to expression of other factors connected with prostate cancer like well-known oncogene *c-ErbB2* (poor prognostic factor) and prostate specific gene *DD3*, highly overexpressed in prostate cancer tissue. In all investigated stages of disease straight dependence between *P65* and *DD3* gene expression and opposite dependence between *P65* and *c-ErbB2* expression were observed [20]. *P65* gene expression was also observed in some cases diagnosed as benign prostatic hyperplasia (BPH) (Balcerczak et al., unpublished data), which may suggest that its appearance may follow transition from benign to adenocarcinoma state. It is already well documented that *H. pylori* causes critical alterations in gastric mucin structure. Long-term bacterial infection is associated with development of gastritis and peptic ulcer and is presumed to be a risk factor for gastric cancer development. The presence of *H. pylori* infection was determined by urease test. Additionally, we had been looking for the presence of *P65* gene transcript in the group of gastric cancers and adjacent normal gastric mucosa. There was no correlation between *P65* gene expression and *H. pylori* infection, which suggests that *H. pylori* is not involved in the process of *P65* gene activation. In the case of gastric cancers *P65* gene expression was connected with poor prognosis for the patients because its expression was detectable in cases with lymph nodes and distant metastases [21]. Taking into consideration that *H. pylori* has been suggested to be a tumor-promoter in gastric carcinogenesis, our earlier results confirmed such hypothesis because anti-*P65* antibodies detected only preneoplastic foci that were tumor-promoter independent [4].

2.2. Qualitative and quantitative analysis of *P65* gene expression in colorectal cancer - comparison with bad prognostic factors

When we analyzed gastric cancer cases, it was first time the positive dependence between *P65* gene expression and poor prognosis for the patients was observed. That is why our

further work was focused on colorectal cancer. Patients with colorectal cancer have the highest mortality among those with cancer. We examined 109 couples of colorectal cancer tissue and adjacent, healthy colorectal mucosa from the same patients and a few samples of colorectal mucosa from patients without colorectal neoplastic disease. For 19 of them the *P65* expression was observed both in cancer tissue and in adjacent colorectal mucosa. In 58 remaining pairs *P65* gene expression was not detected. None of a few cases of healthy mucosa revealed the expression of *P65* gene. Analysis was performed by qualitative technique based on reverse transcription followed by PCR [22] and quantitative analysis by real-time PCR based on fluorescence dye SYBR Green [23]. *P65* gene expression was detected in nearly 50% (n=51) of investigated colorectal cancer cases. There was no statistically significant correlation between age, gender and expression of the *P65* gene. The investigated group consisted of 37 cases of rectal and 72 cases of colon cancers. *P65* gene expression levels determined by quantitative analysis were statistically lower in cancers originating in the rectum as compared to those originating in the colon ($p=0.0099$, Mann-Whitney U test). The analyzed carcinomas were histologically classified as tubular and mucinous adenocarcinomas. There was no statistical correlation between histological type of tumor and *P65* gene expression. There was no statistically significant dependence between *P65* gene expression and histological grade. *P65* gene expression was compared with several clinicopathological parameters (TNM classification) such as depth of tumor invasion (T), lymph node metastases (N), and distant metastases (M). Expression of *P65* was also correlated with vessel invasion and the presence of lymphocytes in tumor tissue. *P65* gene expression was higher in more advanced tumors (T3 and T4, deep wall penetration), while in the T1-T2 group lower levels were recorded. There was statistical dependence between the expression of *P65* and the depth of tumor penetration. Another parameter analyzed was the expression of *P65* gene in cases with (N1-N2) and without (N0) lymph-node metastasis. This analysis also revealed significant statistical correlation between them and, in cases classified as N0, the *P65* expression level was lower than in the N1-N2 cases. Statistically significant correlation was also found between *P65* gene expression and distant metastases. In carcinomas with distant metastases (M1) the levels of *P65* were two times higher than in the group of cancers without distant metastases (M0). The cases without lymphocytes in tumor tissue showed higher levels of *P65* then those with the presence of lymphocytes. Furthermore, in the group of tumors without vessel invasion, *P65* gene expression was lower than in tumors with the vessel invasion, but these differences were not statistically significant [22, 23].

2.3. Influence of anticancer drugs on *P65/p65* expression

In our further studies, we aimed to get information about the possible stimulation of apoptosis and *P65* expression by different compounds in HL-60 cells. For this reason, we have tested the expression of different genes connected with apoptosis like *Bcl-2*, *c-Myc*, *ICE*, *P53* and *Bax* as well as *P65* expression after treatment of human acute promyelocytic leukemia cell line HL-60 by carboplatin alone and in combination with cytoprotective agent amifostine. *Bcl-2*, *c-Myc*, *ICE* and *P53* gene expression was estimated semi-quantitatively using a human apoptosis set 1 detection kit (hAPO1-MPCR) based on multiplex PCR reaction. *Bax* and *P65* gene expression was also estimated semi-quantitatively with the use

of multiplex PCR method where cDNA was generated from total RNA isolated from HL-60 cells with the use of reverse transcriptase. Expression of the investigated genes was determined by normalization to the expression of reference (housekeeping) GAPDH or β -actin genes. HL-60 cells exposed to carboplatin alone showed about 120-fold increase in caspase 3 activity. Combination of carboplatin with amifostine induced the enzyme activity up to 280 times. The level of *Bcl-2* gene expression was diminished, whereas *Bax* gene showed tendency to grow in HL-60 cells treated with carboplatin in combination with amifostine as compared to the cells treated with carboplatin only. Protein product of *Bcl-2* has been shown to block apoptosis in experimental systems and also genetic evidence has indicated that *Bcl-2* suppresses apoptosis. It is known that *Bax* expression is usually connected with induction of programmed cell death. Similarly, to the *Bax* gene, an increasing tendency was also noticed for the *P65*, but its biological role in this process is still unknown [24].

In the same model (HL-60 cells in culture), the influence of amifostine alone and in combination with doxorubicin, cytarabine, or etoposide on *Bcl-2*, *Bax*, and *P65* genes expression was investigated. It was shown that amifostine potentiated cytotoxic action of doxorubicin but not cytarabine and etoposide. HL-60 cells treated with doxorubicin alone showed about 35-fold increase in caspase 3 activity. The enzyme activity was stimulated by combination of doxorubicin with amifostine up to 94 times. Semi-quantitative reverse transcriptase-polymerase chain reaction showed a decrease in *Bcl-2* and an increase in *Bax* and *P65* expression in HL-60 cells treated with doxorubicin in combination with amifostine when compared with the cells treated only with doxorubicin [25].

Expression of the *p65* gene was also analyzed in experimental colon cancer under the influence of 5-fluorouracil (5-FU) given alone and in combination with hormonal modulation. It was found that in the control group (mice bearing colon 38 cancer without treatment) the expression of *p65* gene was present in 57% of investigated samples. In the groups treated with tamoxifen (TAM) or lanreotide (LAN) *p65* expression was detected in 87.5% and 83.3% of analyzed cases, respectively. Both these substances increased apoptotic index in colon 38 cancer as determined by TUNEL method, and a tumor mass. After combined treatment with TAM and LAN a percentage of *p65* positive cases in the cancer group was similar to that of the control group and equal to approximately 60%. This treatment did not increase proapoptotic effects of these drugs used individually. In the group treated with 5-FU and LAN *p65* gene expression was also close to the control value (about 66%). Similarly in this group the combined treatment with these two drugs did not cause any favorable effect on apoptosis. In the group treated with 5-FU alone the expression of *p65* was present in about 80% of samples and increased apoptotic index was observed. In the group treated with a combination of 5-FU and TAM, all analyzed cases exhibited *p65* gene expression [26].

On the basis of these studies, we can conclude that different types of *in vitro* treatment of HL-60 cells, as well as mice bearing colon 38 cancer treated *in vivo*, which leads to apoptosis, is connected with stimulation of *p65/P65* gene expression.

2.4. Cloning, sequencing and chromosomal localization of *P65* gene

Our next goal was to clone and sequence human *P65* cDNA and analyze its expression in malignant tissues. The 5'-RACE and 3'-RACE with previously designed primers [17] were carried out to obtain the 5' and 3' missing portions of the cDNA, respectively. Amplification product of about 900 bp was obtained in 5'-RACE only and cloned in pCR 2.1. Fourteen clones were obtained and gene inserts in seven of them sequenced. The complete cDNA of the human *P65* gene was compiled by overlapping the sequences of cloned 5'-RACE PCR products. The human *P65* transcript consisting of 921 bp was deposited in GenBank Acc No *FJ194940.1*. and, from that time, the *P65* name was replaced by *FJ194940.1*. [27]

BLAT (BLAST-Like Alignment Tool) located the cloned cDNA sequence on human chromosome 1. The cloned sequence spans 2150 bp genomic DNA and consists of five exons. The sequence of intron 1 and intron 4 does not match human genomic sequence. The best matches for intron 1 covered only 37% of sequence in length. Better results were obtained in the BLAT search against the Human Endogenous Retrovirus Database HERVd [28]. Intron 1 sequence was similar to rv_001141. This is a complete typical provirus (soloLTR) with TSD belonging to family HERVL66. Provirus rv_001141 is located on chromosome 1. The most probable explanation is that an integration of additional copy of rv_001141 took place. Part of intron 4 sequence shows similarity (52% coverage, 83% identity) to the second intron of *MLLT3* (NW_001839149).

Integration of an additional copy of rv_001141 changed transcription in this chromosome region. In normal human tissues, this chromosome region (224789942 – 224797092) corresponds to an uncharacterized transcript (GenBank Acc No AK055856) and three EST: DA627076, DA625438, and DA627327. All of them were found in kidney [27].

Chromosomal localization of cDNA of the human *FJ194940.1*. gene was experimentally determined with the use of CHORI-17 (Hydatidiform Mole) Homo sapiens BAC Library from BACPAC Resource Center (<http://bacpac.chori.org>) and was shown to be located on chromosome 1: 224792167-224794166. On the basis of established sequence 40 bp long synthetic probe for *FJ194940.1*. was designed to target a conserved sequence ~900 bp upstream of exon 1. Hybridizations with a ³²P-labeled probe were performed with high-density colony filters containing clones from chromosome 1 segments. The library was hybridized using radioactive probes. Clones from probe-positive well positions were recovered from freezer archives and grown in liquid culture (Balcerczak, Mirowski unpublished data).

2.5. Possible splice variants for *FJ194940.1* gene transcript

To confirm the exon-intron structure of *FJ194940.1* gene generated by the bioinformatics program, eight pairs of primers were designed (Table 2). Four of these primers corresponded to the interior sequences of potential exons. They were denoted as II, III, IV and V. The other corresponded respectively to 3' end and 5' end of adjacent exons. They were denoted as I/II, II/III, III/IV and IV/V [29].

Exon	Primer sequence		Expected amplicon length
	Forward primer	Reverse primer	
I/II	TGGTGTCTATGGAATGCAG	CAGTTCTTCTGGCCCATCCT	153
II	CACTAGAAAACCCTATGACTTTC ACA	GGACAGTTACTTGCAGTTCTT CTG	103
II/III	TGTGAAACAAGCAGTGCAAC	GCTTGTTGGTCAGCCTTCTG	155
III	TTTTCATGTTGATGCTCA	CGCCTGAGTCTGCAGTAAT	101
III/IV	TGCTCATGCATCTCTGCTTT	TTTGAAATGGGAGCCACTGT	218
IV	CAGTGGCTCCCATTTCAAAG	TCAAACAGGTGATCGTTCCA	189
IV/V	CAGCTGGCCTAATCGAAAGA	TCCAGCATTCAGCAAGAGA	155
V	CTCTCTTGCTGAAATGCTGG	GGCCCAGGCTTTAAACTATA	94

Table 2. Primer sequence specific for potential exons and exon junctions of *FJ194940.1* gene and expected product size

The preliminary study has indicated that *FJ194940.1*. gene transcript probably has different splice variants. The mRNA expression of *FJ194940.1* was determined in various types of cancers (breast n =25, colon n=30, thyroid n=25, and acute and chronic leukemias n=50) by semi-quantitative RT-PCR. Amplification conditions were established by gradient PCR. Messenger RNAs for exon III as well as for junction of III/IV and IV/V were found in all investigated samples. PCR product with primers set for exon V was observed in all types of cancers, but it occurred irregularly. Also irregularly, but only in some types of cancer (breast cancer, colon cancer and acute leukemia) products appeared from exon II and IV and junction of II/III. Additionally, it was proven that there is no statistically significant correlation between the presence of exon V and clinical stage and/or cancer grade.

2.6. Detailed investigation of *FJ194940.1* gene alternative splicing in colon cancer

A detailed analysis of 102 colon cancer cases was conducted to confirm an initial observation that pre-mRNA of *FJ194940.1*. gene has undergone alternative splicing. Using the method developed earlier and the above mentioned sets of primers, a total of 18 forms of mRNA *FJ194940.1* splice variants were identified. They have appeared with differential prevalence. Those isoforms arise from different combinations of 4 interior parts of exons (II, III, IV, V) and 4 different exon-exon junctions (created by 3' and 5' ends of particular, adjacent exons I/II, II/III, III/IV and IV/V). The full-length transcript, which was defined as exons I-V and all exon-exon junctions, was seen as the most prevalent in colon cancer samples, being identified in 40 of 102 (39.2%) cases. On the other hand, taking all detected variants (rest of the 17 types of isoforms) into account, they were found in 62 of 102 (60.8%) of patients. Some variants were not observed more frequently than full-length transcripts [29].

The variants of *FJ194940.1* gene are characterized by the lack at least one, as well as the lack of two, three or four investigated elements simultaneously in this gene transcript.

- 5 of the 18 *FJ194940.1* isoforms represented the lack of only one investigated component. Those types of isoforms were detected in 33 of the 102 colon cancer cases (32.4%). The most frequent single omitted element was the junction of II/III exons (16.7%), whereas the most infrequently isolated omitted element was the junction of III/IV as well as IV/V exons (1.9% and 1.9% respectively).
- Two elements were missing in 6 out of 18 *FJ194940.1* isoforms. Those types of isoforms were detected in 18 of the 102 colon cancer cases (17.6%). All but 2 of these variants had omissions of interior sequence of exon with the deleted region of exon-exon junction, i.e. a) II and II/III, b) III and III/IV, c) IV, IV/V, d) II/III, III.
- Three elements were missing in 4/18 *FJ194940.1* isoforms. Those types of isoforms were detected in 7 of the 102 colon cancer cases (6.9%).
- Four elements were missing in 2/18 *FJ194940.1* isoforms. Those types of isoforms were detected in 4 of the 102 colon cancer cases (3.9%). One of these isoforms was missing the entire exon IV.

To sum up the results mentioned above, the splice variant with one element deleted is the most prevalent isoform of *FJ194940.1*. The most frequently omitted element of *FJ194940.1* gene transcript was the junction of II/III exons (36/102, 35.3%). This junction of exons was missing less frequently than in combination with other elements of *FJ194940.1* cDNA. The most infrequently skipped element was the junction of IV/V exons (5.9%). This was incidental to the lack of an interior sequence in exon IV. All samples contained amplification products for the junction of I/II exons and exon V. All possible splice variants of *FJ194940.1* gene are presented on Figure 1 and number of cases with the presence and absence of analyzed splice variants are presented in Table 3.

Additionally, to assess applicability of *FJ194940.1* splice variants elements as prognostic factors in colon cancer, their expression with established prognostic features and survival time of patients suffering from this disease were also compared. Statistical analysis was carried out with reference to whole *FJ194940.1* transcript and to particular exons and exon-exon junctions, but no significant correlations were found. The whole transcript was divided into two parts A and B. The part A consists of exons II and III as well as I/II, II/III exon-exon junctions, while part B is composed of exons IV, V, III/IV, and IV/V exon-exon junctions. Part A contains the most frequently skipped element of the examined transcript, while part B contains its longest component.

No associations were found between gender, familial history and expression of both examined parts of *FJ194940.1* gene transcript. Expressions of both parts A and B were not connected with tumor localization. There was not a statistically significant association between part A and B of *FJ194940.1* gene expression and histological type of tumor. There was link between part B of *FJ194940.1* gene transcript expression and tumor grading. Expression of part B is connected with well (G1) and moderately (G2) differentiated cases. Expression of both parts of investigated gene transcript and some clinical staging features, including depth of tumor invasion, lymph nodes metastases, distant metastases, and stage, according to pTNM classification, were compared. No statistically significant associations were noted between mentioned parameters and expression of part A as well as part B of

FJ194940.1 gene transcript. The expression levels of analyzed parts of *FJ194940.1* gene transcript were also compared to the presence of lymphocytes in tumor and vascular invasion. Venous invasion was significantly related to presence of all elements in part A of the *FJ194940.1* gene transcript ($p=0.0477$). There was no statistical significance connection between the presence of lymphocytes in tumor and expression of part A or B of *FJ194940.1* gene transcript. There was no statistically significant difference in survival time comparing patient with presence and absence of whole transcript in spite of the visible tendency for shorter survival of those without at least one element in whole transcript ($p=0.0690$).

	I/II	II	II/III	III	III/IV	IV	IV/V	V
40	-----	-----	-----	-----	-----	-----	-----	-----
17	-----	-----		-----	-----	-----	-----	-----
8	-----	-----	-----		-----	-----	-----	-----
7	-----			-----	-----	-----	-----	-----
6	-----	-----	-----	-----			-----	-----
4	-----		-----	-----	-----	-----	-----	-----
2	-----	-----	-----	-----		-----	-----	-----
2	-----	-----	-----	-----	-----	-----		-----
2	-----	-----	-----	-----	-----			-----
2	-----				-----	-----	-----	-----
2	-----	-----		-----				-----
2	-----	-----		-----			-----	-----
2	-----			-----			-----	-----
2	-----			-----	-----		-----	-----
1	-----	-----			-----	-----	-----	-----
1	-----	-----	-----				-----	-----
1	-----	-----		-----		-----	-----	-----
1	-----		-----	-----	-----		-----	-----

Figure 1. 18 possible splice variants of *FJ194940.1* gene in colorectal cancer cases (n=102). From the left - the number of cases where splice variants were detected. Amplified exons II, III, IV and V and their junctions I/II, II/III, III/IV, IV/V.

	Number of cases with the presence of investigated amplicon	Number of cases with the absence of investigated amplicon
I/II	102 (100,0%)	0 (0,0%)
II	84 (82,4%)	18 (17,6%)
II/III	66 (64,7%)	36 (35,3%)
III	90 (88,2%)	12 (11,8%)
III/IV	86 (84,3%)	16 (15,7%)
IV	84 (82,4%)	18 (17,6%)
IV/V	96 (94,1%)	6 (5,9%)
V	102 (100,0%)	0 (0,0%)

Table 3. The expression frequency of investigated exons and their junctions in investigated transcript of *FJ194940* gene.

2.7. From cDNA to amino acid sequence

ORF (Open Reading Frame) Finder found several open reading frames on cDNA, which could code protein(s). Sequences of generated peptides were compared by BLAST with sequences of proteins available in GenBank and SwissProt databases. The best results were obtained for ORF number 2 in reading frame 2 on the direct strand extending from base 374 to base 790 (Balcerczak and Mirowski unpublished data).

The peptide amino acids sequences were found to be significantly similar to the integrase core domain in the Conserved Domain Database, and to the human protein EAW69787 referred as similar to Pro-Pol-dUTPase polyprotein, RNaseH, dUTPase, integrase, protease and reverse transcriptase (LOC768966) in the GenBank database. The deduced peptide has a molecular weight of 15.74 kDa and is probably one of possible *FJ194940.1* gene protein products. This hypothesis is in agreement with results that confirmed alternative splicing of the investigated gene. It is also possible that the TAA stop codon at the 790 position is skipped and protein synthesis further continued.

2.8. Anti-peptide antibodies

The deduced protein (15.74 kDa) is substantially smaller than P65. For experimental evidence of this protein sequence potential antigenic region(s) were searched for future experimental analysis. The EMBOSS (European Molecular Biology Open Software Suite) ANTIGENIC program, Kolaskar and Tongaonkar and Peptide Select Program were used. Sequences predicted as antigenic by all the software were used for induction of antibody production. Three peptides (IC14, CL14 and CR14) were chosen for further experiments. Peptides were modified by adding cysteine to the C-terminus or N-terminus and conjugated with KLH. Obtained antibodies were used in Western-blotting analysis of two cell lines: promyelocytic HL-60 and lymphoblastic NALM-6. Positive results were obtained for antibodies IC14 and CR 14 and, in Western blot analysis, two immunoactive bands were detected. The first one had a molecular weight of about 65 kDa and the second about 15 kDa (Mirowski, Balcerczak unpublished data).

2.9. Possible mechanism of transcription in human neoplasm

Sequence of *FJ194940.1* fragment determined in this study had the length of 921 bp. An insertion of an additional copy of HERV provirus rv_001141 probably occurs during the carcinogenic process, which changes transcription of chromosome 1 224792176 - 222794166 region. In normal human tissues this chromosome region encodes transcript AK055856 expressed in kidney. Integration of rv_001141 leads to alteration of transcription [27].

It is already well known that the retrovirus family is an etiological agent of human cancer, mainly leukemia and lymphomas. The presence of endogenous retroviruses (HERV) sequences in the human genome is well documented. In contrast to the normal tissues, HERV sequences are transcriptionally-active in embryonic cells, such as placenta and theraiocarcinoma [30] as well as in tumor cells [31]. Several factors regulate HERV expression. In tumor cells, HERV expression can be stimulated by INF- α [32], TNF- α , IL-1 α and IL-1 β [33]. Other factors, which can stimulate their transcription, are steroid hormones, including glucocorticoids. HERV-K10 (in breast cancer cells) belongs to the family of sequences that are activated in placenta by steroids. On the other hand, HERV-E expression is hindered during therapy with the use of steroids [34]. Another group of factors, which can influence activity of endogenous retroviruses, are products of exogenous viruses e.g., Epstein-Barr, which upregulate transcription of *env* gene of HERV-K18, which is located in the intron of the *CD48* gene [30]. Endogenous retroviruses have influence on genomic DNA organization and deregulate genes expression, creating additional transcription initiation sites, incorrect splicing, inserting stop codons or or polyadenylation signal [30,32,35]. As a consequence of interruption of gene function, many diseases can develop e.g., cancer, if HERV insertion is present in a proto-oncogene or suppressor gene or their vicinity. Such situation is observed in rats with N-methyl-N nitrosourea induced mammary gland carcinoma, where defective sequence of HERV in *c-H-ras* gene intron leads this gene to overexpression [36]. In our previous study P65 antigen was found in the serum and urine of rats with N-methyl-N-nitrosourea-induced mammary adenocarcinomas [5].

3. Conclusion

FJ194940.1 and its protein product seem to be a potential new molecular marker, which may be used for monitoring of oncological patients, especially with colon cancer. Its chromosomal localization and exon-intron structure was predicted by BLAT, confirmed by RT-PCR and also with the use of CHORI-17 (Hydatidiform Mole) Homo sapiens BAC Library from BACPAC Resource Center analysis. The best matches in cloned sequence of *FJ194940.1* gene were found for the Human Endogenous Retrovirus Database HERVd, especially with the family of HERVL66. RT-PCR analysis with sets of primers designed for different exons and junctions of exons, showed alternative splicing of mRNA *FJ194940.1* in different tumors. It leads to production of different *FJ194940.1* isoforms that are expressed with differential prevalence in colon cancer cases. Established nucleotide sequence of *FJ194940.1* gene opens the possibility for further studies with the use of labeled probes or polyclonal / monoclonal antibodies raised against recombinant proteins and such investigations are in progress.

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